*MECHANISM OF ACTION OF STREPTOMYCIN IN E. COLI: INTERRUPTION OF THE RIBOSOME CYCLE AT THE INITIATION OF PROTEIN SYNTHESIS**

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Since 30*S* ribosomes are modified in bacteria resistant to streptomycin,[1, 2] the drug probably affects and kills sensitive cells through an interaction with ribosomes.[3] Studies based on current understanding of the ribosome cycle in protein synthesis have enabled us to show that streptomycin blocks this cycle at a specific point. The block can account for the drug's bactericidal effect.

Mangiarotti and Schlessinger[4] studied the metabolism of polyribosomes in growing cells and suggested the following cycle of ribosome function. Fully formed 30*S* and 50*S* particles exist either in polyribosomes or in a pool of free particles. Periodically, one 30*S* and one 50*S* ribosome join together in an initiation complex on a molecule of messenger RNA (mRNA). The 30*S*-50*S* couple (i.e., 70*S* "monomer") moves across the mRNA as a protein chain is formed. The coupled ribosomes dissociate upon the completion of the protein, and the subunits (30*S* and 50*S*) return transiently to the pool of free particles. This cycle has been supported and elaborated by subsequent experiments with whole cells[5–7] and with subcellular fractions.[8–12] In the present work, we have found that in sensitive cells or in extracts of such cells streptomycin interrupts the ribosome cycle at the initiation of protein synthesis.

Materials and Methods.—Growth of *Escherichia coli* mutant *sud* 24 in fragile form,[13] lysis conditions, labeling conditions to observe long-labeled C^{14}-RNA and pulse-labeled H^3-RNA, and sucrose gradient analyses were carried out as previously described.[4, 5] Growth of strains AB301 (met^-) and N21 (a streptomycin-resistant derivative), grinding with alumina, and incorporation of C^{14}-amino acids *in vitro* were carried out according to Tissières, Schlessinger, and Gros.[14] Total RNA was extracted and tested for mRNA activity by the procedure of Monier *et al.*[15] Growth of RNA phage R17 on host AB301, purification of viral RNA, and incorporation of amino acids directed by the viral RNA in extracts depleted of endogenous mRNA by preincubation were carried out as recommended by Capecchi.[16, 17] H^3-ala-transfer RNA (tRNA) and S^{35}-formylmethionyl (fmet)-tRNA were prepared, and the aminoacyl residue of the unformylated fraction of the S^{35}-met-tRNA was removed according to Marcker.[18] Poly AUG (a 1:1:1 random copolymer) was a Miles Laboratory product. Streptomycin sulfate was obtained as a 50% solution from Eli Lilly and Co., stored at 4°C, and diluted just prior to addition to growing cultures or incorporation mixtures. C^{14}-uracil (33 mc/mmole), H^3-uracil (23–27 c/mmole), C^{14}-L-alanine (91 mc/mmole), and S^{35}-L-methionine (1.49 c/mmole) were all from Schwarz BioResearch, Inc., Orangeburg, New York.

Results.—(a) *Polyribosome metabolism in cultures treated with streptomycin:* Addition of streptomycin to a growing culture of *E. coli* leads to a rapid and decisive interruption of protein synthesis (see review by Brock[19]). To determine how the drug disrupts polyribosome metabolism, we have made use of a strain growing in fragile form,[13] which is rapidly and easily lysed and thereby facilitates the detailed analysis of ribosome distributions.[4, 5] The ribosomes were labeled with C^{14}-uracil and their size distribution was analyzed in sucrose gradients at intervals after streptomycin addition.

A typical example of the changes is seen in Figure 1 (*solid lines*), which shows characteristic analyses of cell extracts made just before addition of streptomycin, and 2 and 40 minutes thereafter. At the moment of addition of streptomycin, ribosomes were found as free 30*S* and 50*S* particles, as well as bound to mRNA. The successive faster-moving peaks correspond to mRNA molecules bearing one, two, three, etc., ribosome couples. After addition of the drug, protein synthesis was rapidly inhibited (more than 90% within 10 min), and the following changes in ribosome distribution occurred: (1) a decrease in large polyribosomes and in free 30*S* and 50*S* particles; and (2) a striking *accumulation of 70S monomers* (i.e., single ribosome couples).

Pulse-labeled H^3-RNA (Fig. 1, *dotted lines*) continued to be associated with the remaining polyribosomes and 70*S* monomers. However, the ratio of H^3/C^{14} in 70*S* monomers compared to that in larger polyribosomes gradually declines (Fig. 1); i.e., the relative rate of appearance of new RNA on monomers decreased with time. The decreasing ratio indicates that the 70*S* monomers are a special class of ribosomes and do not arise from random fragmentation of polyribosomes during extraction, since monomers obtained by fragmentation would have the same H^3/C^{14} ratio as polyribosomes.[4] In a forthcoming paper (manuscript in preparation), we will show that each accumulated monomer bears a molecule of stabilized mRNA. Therefore, when a pulse label is given, only the monomers that accumulate during the pulse period can bear newly formed mRNA. The monomer region thus shows an increasingly marked deficit of H^3-pulse label.

These results suggest that streptomycin retards the movement of ribosomes, specifically at or near the beginning of the mRNA molecule, and thereby stops protein synthesis. We visualize that each time a 30*S*–50*S* couple containing bound streptomycin forms on a molecule of mRNA, the couple is held up at the starting point and is unable to engage in protein synthesis; but monomers that have already started a polypeptide chain can proceed to the end of the mRNA relatively unimpeded. Ribosomes are thereby redistributed on mRNA, and mRNA molecules bearing one or only a few 30*S*-50*S* couples accumulate. Studies of the effects of streptomycin in cell-free systems, summarized below, strongly support this model.

(b) *Streptomycin blocks the function of natural mRNA:* Although streptomycin causes rapid cessation of protein synthesis in intact cells, the inhibition of protein synthesis in extracts has been much less pronounced. When either polyuridylic acid or other artificial mRNA is used, the drug produces only partial inhibition (0–80%).[20–22] We have found, however, that this discrepancy between the effect of streptomycin in whole cells and in extracts disappears if natural mRNA is used instead of artificial mRNA molecules such as poly U or poly A. For example, protein synthesis directed by RNA from phage R17 was *completely* blocked by streptomycin at a level of one molecule of drug per ribosome (Fig. 2). (At 0.3, 0.5, 1, and 3 μg/ml streptomycin, the observed inhibition was 8, 48, 94, and 100%, respectively; since 1 ml of reaction mixture contains 2–3 mg ribosomes (0.8–1.2 mM 30*S* and 50*S* particles), 1 μg/ml streptomycin (1.4 mM) corresponds to about one molecule per ribosome.) In contrast, extracts of resistant strains responded to R17 RNA just as well in the presence of the drug as in its absence (Fig. 2). The functioning of natural mRNA ex-

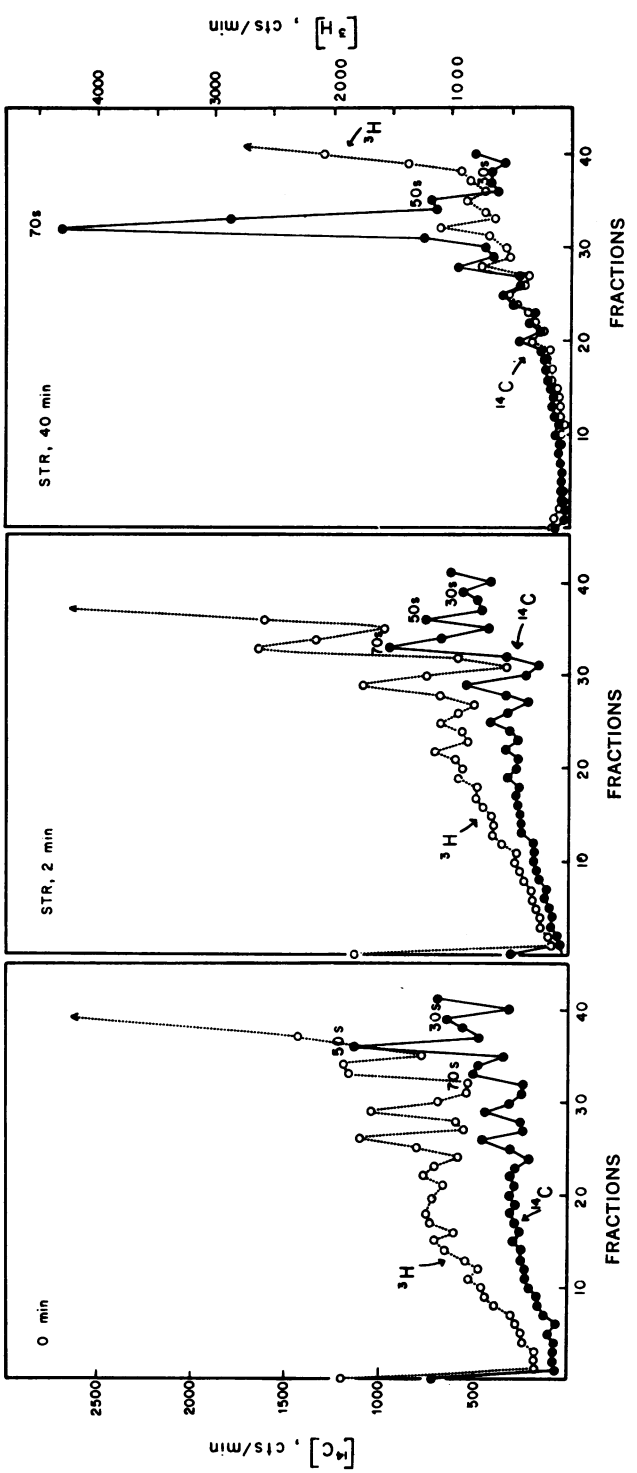

FIG. 1.—Disappearance of polyribosomes and accumulation of 70S monomers in sensitive cells treated with streptomycin. A 50-ml culture of strain *stud* 24 was grown in fragile form and stable RNA prelabeled with C^{14}-uracil (3 μc in 0.1 μmole). After two generations of growth, streptomycin (75 μg/ml) was added to the culture (similar results were obtained using 10 μg/ml streptomycin). At 0, 2, and 40 min, 10-ml portions of culture were withdrawn; each was then pulse-labeled for 90 sec with H^3-uracil (20 μc in 0.006 mmoles). Each portion was centrifuged, the cells were lysed,[4] and the lysates analyzed by zonal sedimentation in 35 ml 15–30% sucrose gradients. Centrifugation was for 3 hr at 25,000 rpm in an International B-60 ultracentrifuge with the SB-110 swing bucket rotor at 5°C. Each gradient was collected in 45 fractions, and trichloroacetic acid-insoluble H^3 and C^{14} were measured in a Packard Tri-Carb liquid scintillation counter.
(●——●) C^{14}; (O···O) H^3.

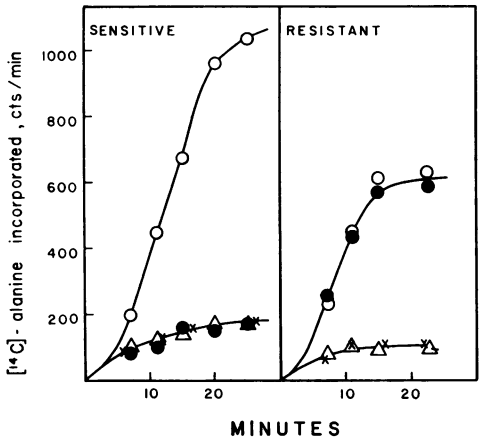

FIG. 2.—Streptomycin blocks R17 phage protein formation in extracts of sensitive strains, but not of resistant strains. Protein synthesis was directed by phage RNA in preincubated extracts of strain AB301 (*at left*) and strain N21, a streptomycin-resistant derivative (*at right*). For technique, see *Materials and Methods*.

(O——O) 500 μg/ml R17 RNA added; (●——●) 500 μg/ml R17 RNA and 1 μg/ml streptomycin added; (×——×) no R17 RNA added (Δ——Δ) no R17 RNA, 1 μg/ml streptomycin added.

The values obtained at time 0 (20–30 cpm) were subtracted from the values obtained at later times.

tracted from *E. coli* and then added to preincubated extracts from sensitive cells was equally sensitive to streptomycin. Thus, in extracts prepared from a streptomycin-sensitive strain, as in whole cells, protein synthesis directed by natural mRNA can be completely abolished by streptomycin.

(c) *Streptomycin blocks normal initiation of protein synthesis:* Why does streptomycin block protein synthesis directed by natural mRNA, but not that directed by the artificial mRNA's such as poly U, poly A, and poly C? The most critical difference between the two kinds of RNA is known to be that natural mRNA initiates protein synthesis by a mechanism requiring the apposition of fmet-tRNA to the corresponding AUG codon.[17] In contrast, poly U initiates protein synthesis by a mechanism that seems to require distortion of a ribosomal binding site such as occurs in the presence of elevated concentrations of Mg^{2+}.[8, 23] These considerations, independent of the accumulation of 70*S* monomers observed in intact cells (section (*a*)), also suggest that streptomycin inhibits the normal initiation of protein synthesis.

If streptomycin selectively inhibits natural (fmet-tRNA-dependent) initiation of protein synthesis, then (1) initiation that is not natural might not be blocked; (2) protein synthesis directed by mRNA already functioning on ribosomes should be relatively uninhibited; and (3) the initiation process should be specifically blocked by streptomycin, presumably before the formation of the first peptide bond.

The following observations show that all these expectations have been fulfilled. (1) At high levels of Mg^{2+}, initiation with natural mRNA can occur by an abnormal mechanism.[8, 23] Streptomycin is still bound, for its activity in promoting miscoding is increased at high concentrations of Mg^{2+};[24] nevertheless, protein synthesis directed by phage RNA (Fig. 3), as well as by the endogenous mRNA made in cell extracts,[17] escapes the inhibitory action of the drug. Streptomycin inhibition of the protein synthesis directed by phage RNA decreased from 93 to 37 per cent as the Mg^{2+} concentration was raised from 5 to 20 mM. In similar experiments with natural cellular RNA, inhibition decreased from 80 per cent to 15–20 per cent.

Comparable results are seen for the stimulation of protein synthesis by poly

AUG, which contains the natural initiating codon AUG. Protein synthesis carried out at 7 mM Mg^{2+} or less is completely inhibited by streptomycin, but streptomycin has no inhibitory effect at 20 mM Mg^{2+} (Fig. 4), where the variant mechanism of initiation is possible.

(2) The completion of polypeptides by endogenous mRNA already on ribosomes in a DNase-treated extract[17] is very insensitive to streptomycin (Fig. 2 (samples with no added R17 RNA) and unpublished results).

(3) R17 RNA directs the binding to ribosomes of fmet-tRNA and of ala-tRNA, which correspond to the first and second amino acids incorporated into protein. The amount of tRNA bound in the presence and in the absence of streptomycin was estimated in sucrose gradients. The effect of the drug was unequivocal: streptomycin sharply reduced the amount of S^{35}-fmet-tRNA and H^3-ala-tRNA bound to ribosomes (to 15% and 0–6% of the control, respectively; Table I). In a corroborative experiment, protein synthesis was directed by R17 RNA, and the formation of the dipeptide fmet-ala was gauged by the technique of Ghosh and Khorana.[12] The amount of fmet-ala formed in the presence of streptomycin was less than one tenth the amount formed and incorporated into acid-insoluble protein chains in the absence of the drug.

Discussion.—(a) *Mode of action of streptomycin:* On the basis of the fore-

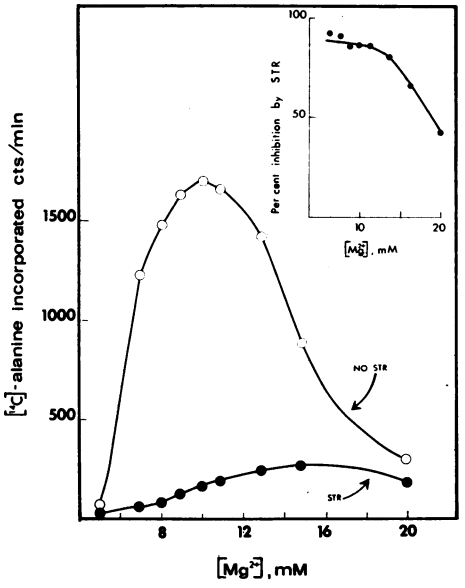

FIG. 3.—Effect of streptomycin on R17 RNA-directed protein synthesis as a function of Mg^{2+} concentration. A preincubated extract of AB301, supplemented with 500 μg/ml R17 RNA, was tested at different Mg^{2+} concentrations. Incubation time: 20 min.

(O——O) no streptomycin; (●——●) with 2 μg/ml streptomycin.

In the inset, the inhibitory effect of streptomycin is plotted as a function of Mg^{2+} concentration.

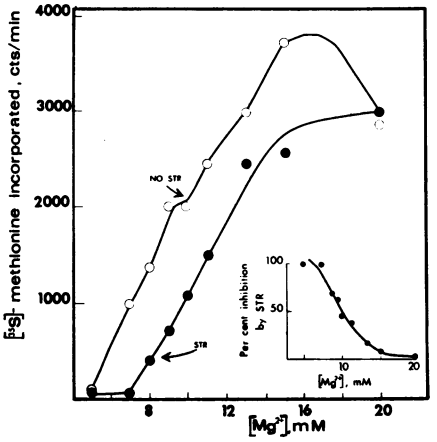

FIG. 4.—Effect of streptomycin in poly AUG-directed protein synthesis as a function of Mg^{2+} concentration. The preincubated extract was supplemented with 20 μg/ml poly AUG; S^{35}-methionine was used. Incubation time: 20 min.

(O—O) no streptomycin; (●—●) with 2 μg/ml streptomycin.

Inset as in Fig. 3.

going experiments, we suggest that streptomycin alters the normal interaction of sensitive 30*S* and 50*S* ribosomes on mRNA,[25] and that this leads to the appearance of "streptomycin monomers," i.e., ribosomes blocked as *aberrant initiation complexes*. The modified complexes cannot form peptide bonds, and their movement along the messenger is impeded. Hence the addition of further ribosomes is inhibited and the normal cycle of ribosome function is interrupted. Though the modified initiation complex does not form protein, streptomycin does not prevent the completion of growing peptide chains. Therefore, as more and more ribosomes bind streptomycin in the cell during the initiation process, polyribosomes gradually decrease in size until there tends to be only a pseudo-initiation complex (70*S* monomer) on each mRNA chain.

According to this view, the mutation to a streptomycin-resistant form alters the way in which 30*S* ribosomes interact with 50*S* ribosomes, so that functional initiation complexes can be formed even in the presence of the drug. Indeed, Leon and Brock[27] have shown that the mutation has a direct effect on the interaction of the subunits; in the absence of the drug, 70*S* ribosomes from sensitive strains showed a greater tendency to hold together at high temperature than did ribosomes from a resistant mutant.

Our present understanding of the ribosome cycle (see introduction) suggests that 70*S* monomers might accumulate with inhibition of protein synthesis either by the stabilization of 30*S*-50*S* couples in a completely abnormal way, or by the blockage of newly formed monomers at protein initiation, or by the blockage of dissociation of the monomers at the termination of a polypeptide chain. Although

TABLE 1. *Streptomycin inhibition of R17 phage RNA-directed binding of aminoacyl-tRNA to ribosomes.*

Additions	tRNA Bound to Ribosomes (cpm) S^{35}-fmet	H^3-ala
Experiment 1		
Complete	720	168
" plus streptomycin	470	55
" minus R17 RNA	420	68
Experiment 2		
Complete	1990	887
" plus streptomycin	550	272
" minus R17 RNA	370	230

Under the general conditions used by Capecchi,[17] tRNA was bound to ribosomes at 36° for 7 min in a preincubated extract of AB301. The "complete" sample contained 0.18 ml of preincubated extract and 200 μg R17 RNA in a final volume of 0.77 ml.[17] Streptomycin, where used, was added at 3 μg/ml to the preincubated extract before other additives. After incubation at 36°, the samples were centrifuged through 15–30% sucrose gradients in 0.04 *M* KCl, 0.01 *M* tris(hydroxymethyl) aminomethane (Tris)-HCl, pH 7.0, and 0.01 *M* magnesium acetate for 4 hr at 24,000 rpm in the SB-110 rotor of the International B-60 ultracentrifuge. Each gradient was run through a Gilford model 2000 absorbance recorder to locate the peak of 70*S* ribosomes and was then collected in 15 fractions. The fractions were precipitated with ice-cold 5% trichloroacetic acid, plated on glass fiber filters, and counted in a Packard liquid scintillation spectrometer. The counts bound to ribosomes in each gradient are tabulated. The sources of label were, in Experiment 1, 18,000 cpm S^{35}-fmet-tRNA and 6,600 cpm H^3-ala-tRNA (180 μg total tRNA) and, in Experiment 2, 26,000 cpm S^{35}-fmet-tRNA and 40,000 cpm H^3-ala-tRNA (340 μg total tRNA). In both experiments, of the radioactivity remaining insoluble in cold acid after the gradient centrifugation, about 40% of the S^{35} and 80% of the H^3 were bound to ribosomes in the complete system; the remainder were in the soluble phase. In Experiment 2, to gauge tRNA binding in the absence of measurable protein synthesis, the Mg^{2+} concentration was reduced from the standard 11 mM down to 7 mM, and unlabeled amino acids, adenosine 5′-triphosphate (ATP), phosphoenolpyruvate, and pyruvate kinase were left out of the reaction mixture. Under these conditions, no detectable label became insoluble in hot trichloroacetic acid.

the detailed mechanism of the accumulation of 70S monomers in intact cells is not analyzed here, these monomers appear to be modified initiation complexes. Among the less likely alternatives are the following: (1) It seems unlikely that streptomycin acts as a bifunctional ligand to "glue" together free 30S and 50S particles to form 70S monomers, because streptomycin itself does not cause these particles to associate in extracts.[26] (2) The 70S monomers do not result from their failure to leave mRNA on completion of a polypeptide chain, for if initiation were not blocked, one would observe an accumulation of large polyribosomes rather than of 70S monomers. (3) It is also unlikely that the 70S monomers represent 30S-50S couples that did not dissociate after polypeptide chain termination and moved undissociated to a new mRNA. If this were the case, streptomycin would not block the initiation of protein synthesis on free 30S and 50S particles. However, initiation of protein synthesis directed by R17 RNA, a process that begins on the free 30S and 50S ribosomes in extracts,[10] is totally blocked by the drug (Fig. 2).

The nature of the blocked initiation complexes is not completely known. In particular, it is not yet clear whether they contain any tRNA, since streptomycin reduces the amount of S^{35}-fmet-tRNA bound in the presence of phage RNA (Table 1). However, 30S-50S couple formation ordinarily requires tRNA,[8] and it may be that fmet-tRNA is bound in a deacylated form, or is deacylated on the abortive complex, releasing the S^{35}-label from the ribosomes. (See *Note added in proof.*)

(b) *A note on killing by streptomycin:* What is the relationship between the effect of streptomycin on the initiation of a protein chain and its well-known effects occurring during the synthesis of a protein chain, manifested either as inhibition[20-22] or miscoding[22, 24, 28] in the translation of certain codons? Since at bactericidal concentrations of 10 μg/ml or higher streptomycin blocks ribosomes at initiation, no protein synthesis then takes place. The interaction of streptomycin with ribosomes located at later codons is then precluded. In other words, neither misreading nor other effects at internal codons seem relevant to the killing action of the drug.[29]

While the accumulation of aberrant 70S monomers with consequent interruption of protein synthesis is *sufficient* to explain the arrest of cell growth, we have no definitive evidence as yet that it represents the primary event in the bactericidal action of streptomycin. However, one simple possibility is that streptomycin binds to ribosomes so tightly that the formation of the aberrant initiation complex is irreversible in sensitive cells and leads to cell death.

Summary.—When lethal concentrations of streptomycin are added to a growing culture, polyribosomes gradually decrease in size and number, and 70S monomers accumulate progressively (to as much as 60% of the total ribosomes by 40 min). The monomers do not incorporate radioactive amino acids into protein. These monomers are probably at the beginning of the mRNA molecules, a suggestion substantiated by the action of streptomycin in cell extracts. One molecule of drug per ribosome completely blocks R17 phage RNA-directed protein synthesis in extracts of a sensitive strain, but not of a resistant strain, at Mg^{2+} concentrations of 7–10 mM where initiation is "natural" (fmet-tRNA-dependent). On the other hand, polypeptide chain elongation can con-

tinue even in the presence of streptomycin if protein synthesis is already beyond the initiation steps before the drug is added, or if initiation is forced on mRNA "unnaturally" (for example, at higher levels of Mg^{2+}). The notion that normal initiation is blocked is further corroborated by the finding that R17-directed binding to ribosomes of S^{35}-fmet-tRNA and H^3-ala-tRNA is severely inhibited by streptomycin. We conclude that the critical action of streptomycin is to modify the association of 30*S* and 50*S* particles to yield aberrant initiation complexes,[8] and that this interrupts the ribosome cycle.[4]

Note added in proof: Subsequent experiments showed that 30*S*–50*S* couple formation in presence of streptomycin still required tRNA as well as Mg^{2+}, K^+, and mRNA.

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